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Mutations in Soybean Microsomal Omega-3 Fatty Acid Desaturase Genes Reduce Linolenic Acid Concentration in Soybean Seeds

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ABSTRACT

One major locus (*Fan*) and several minor loci have been shown to contribute to the linolenic acid level in soybean [*Glycine max* (L.) Merr.] seeds. The *Fan* gene encodes a microsomal omega-3 fatty acid desaturase (*Arabidopsis* FAD3 homolog), and soybeans contain three *FAD3* genes. The objective of this work was to characterize candidate soybean *FAD3* genes from low linolenic acid soybean lines and associate those alleles with the trait. Mutations in two of the three soybean *FAD3* genes were identified, and genotypes with the mutant alleles conferred a reduction of over two thirds of the linolenic acid present in the seed. The two mutant genes contributed unequally but additively to the phenotype. The results demonstrated that the mutant genotype can be identified with mutation-specific molecular markers in the F_2 generation, and the low linolenic acid trait will be stably inherited in subsequent generations.

THE PRODUCTION of soybeans containing low linolenic acid levels in the oil fraction is desirable for the demands of modern markets. Through mutagenesis breeding, a set of soybean lines has been developed with lowered seed linolenic acid levels, and these lines have mutations at the *fan* locus (Wilcox and Cavins, 1985; Rennie and Tanner, 1991; Rahman et al., 1996). Other lines have been described in which lower linolenic acid phenotypes were generated, and the inheritance was characterized as multigenic (Fehr et al., 1992; Rahman et al., 1997; Rahman et al., 1998; Takagi et al., 1999; Ross et al., 2000). Successful incorporation of multiple genes at independent loci into elite lines would be enhanced by the use of molecular markers specific for the mutated genes. Mutation-specific allele markers would also enable multigenic traits to be efficiently selected in lines used for backcrossing into elite lines, decreasing the dependence on segregation of poor agronomic traits inherited from the mutated parent line with the desired trait.

Omega-3 fatty acid desaturase enzymes introduce the third double bond into linoleic acid precursors to produce linolenic acid precursors. We previously confirmed that the *Fan* locus was one of the three soybean homologs (*GmFAD3A*) of the *Arabidopsis* microsomal omega-three fatty acid desaturase gene *FAD3* (Bilyeu et al., 2003). *GmFAD3A* was also characterized as the most highly expressed of the three homologs in developing seeds. The relative importance of *GmFAD3B* and *GmFAD3C* to seed linolenic acid levels has not yet been described, although they have been shown to be expressed at low levels in developing seeds (Bilyeu et al., 2003). Nuclear-encoded, chloroplast-targeted omega-three fatty acid desaturases may also contribute to seed linolenic acid levels (Yadav et al., 1993). Omega-3 fatty acid desaturases are members of an enzyme family characterized by the presence of a diiron cofactor which interacts with three regions of conserved histidine motifs in the protein (Shanklin et al., 1994).

The low linolenic acid soybean line CX1512-44 was previously developed from a mutagenesis breeding program (James Wilcox, pers. comm.). Although dependent on the environmental growth conditions, CX1512-44 seeds typically contain about 3% (30 g kg^{-1} oil) linolenic acid, while wild-type lines contain about 7 to 10% (70–100 g kg^{-1} oil) linolenic acid. On the basis of segregation analysis of the fatty acid composition phenotype of F_2 progeny from crosses between CX1512-44 and lines with wild-type linolenic acid levels, the low linolenic acid trait was presumed to be multigenic (data not shown). A genetic characterization of CX1512-44 has not been described in the literature. The objective of this work was to characterize candidate soybean *FAD3* genes from low linolenic acid soybean lines and associate those alleles with the trait. We previously determined that soybean contained three microsomal omega-3 fatty acid desaturase genes. We confirmed by sequence analysis that the three CX1512-44 *FAD3* genes were candidates for genetic lesions associated with the low linolenic acid trait. In addition, we were able to associate the linolenic acid level phenotype and the *FAD*3 genotype for progeny of crosses derived from CX1512-44.

MATERIALS AND METHODS

Cloning and Sequence Analysis

Primers were designed on the basis of the sequences deposited in the GenBank to amplify soybean *GmFAD3A* (AY204711), *GmFAD3B* (AY204712), and *GmFAD3C* (AY204713) cDNAs from line CX1512-44. Reverse transcriptase reactions, PCR amplification, isolation of products from agarose gels, and cloning were as previously described (Bilyeu et al., 2003).

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Abbreviations: bp, base pairs; CAPS, cleaved amplified polymorphic sequence; dNTP, deoxyribonucleotide triphosphate mix; PCR, polymerase chain reaction; RT, reverse transcriptase; SNP, single nucleotide polymorphism.

Plant Growth

The 3% linolenic acid line 2721 was an F_6–derived line produced from a cross between Pana and CX1512-44. Line 2721 was homozygous for the CX1512-44 mutant alleles of *GmFAD3A* and *GmFAD3C*. Seeds of a 'Williams 82' (Bernard and Cremeens, 1988) × 2721 cross and A5 (Rennie and Tanner, 1991) × 2721 were produced in Costa Rica in 2003. F_1 and F_2 plants were germinated in moist packets and transferred to soil for growth in growth chambers set at 27.5/23°C day/night with 14.5 h daylength. The light intensity was 750 μmol m^{-2} s^{-1}. F_4 seeds were produced in a greenhouse in the spring of 2004 without supplemental lighting.

Phenotype Analysis

For fatty acid determination, chips were made distal to the embryonic axis (approximately 20% of the seed) of individual F_2 seeds and the parent line seeds, and the portions containing the embryonic axis were saved for germination. For the F_3 seed fatty acid determination, 10 F_3 seeds from each F_2 line were crushed and analyzed individually. The only exception was for genotype aaCC, where aa represents mutant alleles at *FAD3A* and CC represents wild-type alleles at *FAD3C*, for which only five seeds were used. F_4 seeds ($n = 3$) from three F_3 plants representing three independent aacc genotypes were crushed and analyzed individually. The concentration of linolenic acid in the sample was determined as a percentage of the total fatty acids of the seed by lipid gas chromatography of fatty acid methyl esters of extracted oil. Crushed seeds were extracted in 0.5 mL (for chips) or in 1 mL (for single seeds) chloroform:hexane:methanol (8:5:2, v/v/v) overnight. Derivitization of 0.5-mL chip solvent or 150-μL single seed solvent was done with 75 μL methylating reagent (0.25 *M* methanolic sodium methoxide:petroleum ether:ethyl ether, 1:5:2, v/v/v). For single seeds, samples were diluted with hexane to approximately 1 mL. An Agilent (Palo Alto, CA) series 6890 capillary gas chromatograph fitted with a flame ionization detector (275°C) was used with an AT-Silar capillary column (Alltech Associates, Deerfield, IL). Standard fatty acid mixtures (Animal and Vegetable Oil Reference Mixture 6, AOACS) were used as calibration reference standards.

Detection of Mutant Alleles

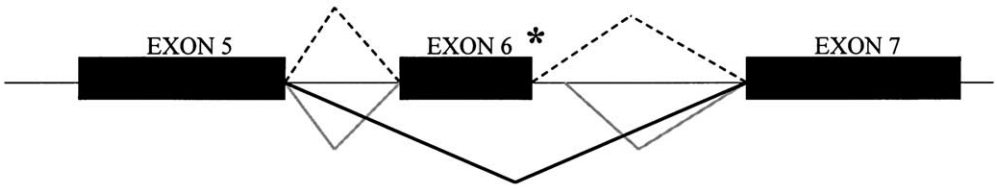
For both the CX1512-44 *GmFAD3A* and *GmFAD3C* alleles, a detection method for SNPs was developed on the basis of the McSNP assay (Ye et al., 2002). The assays are based in principle on cleaved amplified polymorphic sequence (CAPS) assays (Konieczny and Ausubel, 1993), where the genomic region of interest is first amplified by PCR followed by a restriction enzyme digestion which distinguishes the wild-type and mutant alleles. We utilized a real-time PCR instrument (MJ Research Opticon, Waltham, MA) and the double-stranded DNA binding dye SYBR Green I (Molecular Probes, Eugene, OR) for a melting curve analysis so that PCR digestion products could be characterized without separation on agarose gels. For *GmFAD3A*, the primers were 3AD1 (TTGCATCAC CATGGTCATCAT) and 3AIX (AGCTATTATCTAGCA TTAACCTCA). For *GmFAD3C*, the primers were 653Dup (GTCCTTTGTTGAACAGCATT) and 653T (CTCCTGCA AAAAATCCATGAGTTGT). While 3AD1 and 653Dup were derived from cDNA sequences, 3AIX and 653T were derived from intron sequences to increase priming specificity. Scoring for a deletion of *GmFAD3A* was by resolution of one or three products after PCR with primers that allowed amplification of 153 bp of the *GmFAD3A* gene as well as a 165-bp product from the *GmFAD3B* gene using the primers 387start (AGC AATGGTTAAAGACACAAAG) and 387testab (AGGGA TCTCCATGGATTCTTGA). For genotypes with both copies of *GmFAD3A* deleted, a single band was produced, whereas when *GmFAD3A* was present in one or two copies, three bands were produced (one each for *GmFAD3A* and *GmFAD3B* as well as a slower-migrating band that was a hybrid of the two products). PCR templates consisted of 2 mm washed FTA (Whatman, Clifton, NJ) card punches prepared from leaves according to the manufacturer's instructions. The 15-μL reactions for PCR contained template, buffer (40 m*M* Tricine-KOH (pH 8.0), 16 m*M* KCl, 3.5 m*M* $MgCl_2$, 3.75 μg mL^{-1} BSA, 200 μ*M* dNTPs), 10% (v/v) DMSO, 0.5 μ*M* each primer, 0.25× SYBR Green I, and 0.2× Titanium *Taq* polymerase (BD Biosciences, Palo Alto, CA). Amplification conditions were 95°C for 5 min, 35 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 20 s. Restriction enzyme reactions were performed for 14 to 18 h in the same tube after the addition of 30 μL of a mix containing 20 μL 2× *Mae*III buffer (Roche Applied Science, Indianapolis, IN), 9.85 μL ddH_2O, and 0.15 μL *Mae*III (1.67 U μL^{-1}, Roche Applied Science, Indianapolis, IN) for *GmFAD3A* or 3.5 μL 10× buffer 2 (New England Biolabs, Beverly, MA), 26.3 μL ddH_2O, and 0.2 μL *Nco*I (10 U μL^{-1}, New England Biolabs, Beverly, MA) for *GmFAD3C*. *GmFAD3A Mae*III reactions were incubated at 55°C, while *GmFAD3C Nco*I reactions were incubated at 37°C. Melting curve analsyis followed restriction enzyme digestion of PCR products with parameters of 70 to 90°C with 0.2°C increases and reads every 1 s. For *GmFAD3A*, wild-type alleles (100- and 48-bp products) produced a peak at 78°C, mutant alleles produced a peak at 82.5°C (148 bp) and heterozygotes produced both peaks (148, 100, and 48 bp). For *GmFAD3C*, wild-type alleles (134- and 59-bp products) produced a peak at 79.5°C, mutant alleles produced a peak at 81.5°C (193 bp) and heterozygotes produced both peaks (193, 134, and 59 bp). In some cases, products were resolved on 1.5% (w/v) agarose gels.

RESULTS

Characterizing the *CX1512-44 GmFAD3A, GmFAD3B, and GmFAD3C* Genes

Immature seed or leaf tissue from the CX1512-44 line was used as a source of RNA for amplification of cDNA sequences for *GmFAD3A, GmFAD3B,* and *GmFAD3C* (Bilyeu et al., 2003) by reverse transcriptase PCR (RT-PCR). Amplification of subsections of *GmFAD3A* produced products of unanticipated sizes. Sequencing of clones of these products revealed mRNA missplicing events in the 3′ half of the gene, specifically the activation of a cryptic 5′ splice site in one case and the complete exclusion of an exon in another. The amplification of these products supported the exon-definition model of splicing plant introns (Simpson et al., 1999), and pointed to a potential splice-site lesion. Evaluation of the target DNA sequence from genomic DNA, which corresponds to the sixth intron of the *Arabidopsis FAD3* gene, exposed a G to A transition (Fig. 1) in a consensus splice-site sequence (Brown et al., 1996) in CX1512-44 compared with Williams 82. The mutation occurred at the position following base 810 of the coding sequence, in the first base position of the putative sixth intron of *GmFAD3A*. Williams 82 cDNA did not appear to produce any misspliced products when amplified with the primers that revealed missplicing in CX1512-44 (data not shown). Both of the detected misspliced CX1512-44 *GmFAD3A*

A.



B.

Williams 82	CAAG	GTAAC
CX1512-44	CAAG	ATAAC

C.

TTGCATCACCATGGTCATCATCAGAAACTGCCTTGGTATCGCGGCAAG(gt
aac)aaaaataaatagaaaatagtgagtgaacacttaaatgttagatacta
ccttcttcttctttttttttttttt**tgaggttaatgctagataatagct**

Fig. 1. Identification of a splice-site mutation in the CX1512-44 *GmFAD3A* allele. A. schematic diagram of *GmFAD3A* exons (5–7, black rectangles) connected by introns (horizontal lines). Normal splicing and intron removal is represented by dashed lines above the introns. Abnormal splicing (black and gray lines below the introns) characterized CX1512-44 mRNA with either exclusion of exon 6 or activation of a cryptic splice site within an intron leading to improper expansion of exon 6 coupled to proper splicing of exons 5 and 7. A mutation in the splice site was identified at the first base following exon 6, (position denoted by an asterisk). B. Sequence comparison of Williams 82 and CX1512-44 *GmFAD3A* splice site following exon 6. PCR products generated from amplification of genomic DNA were cloned and sequenced. Sense sequence is listed 5′ to 3′, with the vertical line indicating the separation of exon 6 and intron sequence. The first base of the intron in the CX1512-44 allele is changed from G to A (underlined), mutating a consensus splice site ($G^{XXX}GT$, bold, Brown et al., 1996). C. Sequence of genomic DNA from Williams 82 *GmFAD3A* beginning with base 763 of the cDNA sequence and including the exon 6/intron 6 boundary. Exon sequences are listed in capitals while intron sequences are lowercase. The sequences corresponding to the PCR primers used for the *GmFAD3A Mae*III assay are shown in bold (see Materials and Methods). The base mutated in CX1512-44 is underlined, and the *Mae*III recognition sequence (GTNAC) is in parentheses.

mRNAs would produce prematurely terminated protein sequences resulting from frameshifts in the coding region. Therefore, the single base mutation in the CX1512-44 *GmFAD3A* gene renders the desaturase enzyme nonfunctional.

The *GmFAD3B* gene from CX1512-44 was also queried for putative mutations. While several polymorphisms were identified when the CX1512-44 *GmFAD3B* gene was compared with the genes from 'Pana' (Nickell et al., 1998) and Williams 82, no highly conserved amino acids were affected by the changes. We assumed that the CX1512-44 GmFAD3B enzyme was functionally equivalent to a wild-type enzyme.

The CX1512-44 *GmFAD3C* gene was similarly cloned and sequenced. A single nucleotide polymorphism (SNP) was identified at base 383 (a383g) of the coding sequence that alters a codon for glycine to glutamic acid (G128E) within the conserved histidine-rich region 1b of the enzyme (Shanklin et al., 1994) (Fig. 2). When the mutant peptide fragment was used as a query in a search of GenBank (tblastn), all of the identified sequences contained a glycine in the CX1512-44-mutated amino acid position. Although the deleterious effect of this particular amino acid change has not been independently demonstrated biochemically, the high degree of sequence conservation implies functional significance of the region.

Fig. 2. Amino acid alignment of a portion of the soybean and *Arabidopsis* FAD3 protein sequences. Identical amino acids are highlighted in black while similar amino acids are highlighted in gray. The wild-type and CX1512-44 GmFAD3C (CX1512-44C) alleles are shown from amino acid 103 to 152. A SNP in the coding sequence results in a G128E mutation (indicated above the alignment with an asterisk) for the CX1512-44 allele. Histidine-rich region 1b (Shanklin et al., 1994) is underlined.

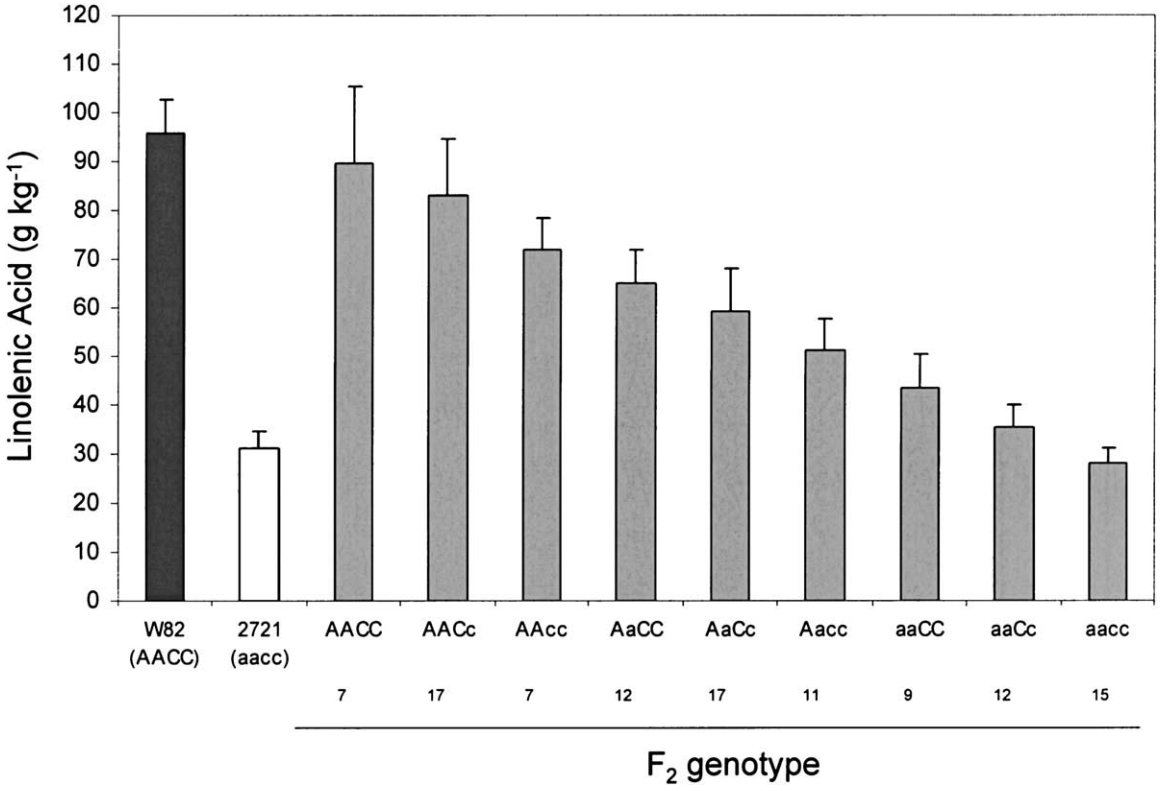


Fig. 3. Phenotype and genotype for F_2 plants segregating for *GmFAD3A* and *GmFAD3C* mutations. Histogram representing the linolenic acid phenotype of F_2 soybean seeds segregating for the *GmFAD3A* and *GmFAD3C* mutations from a cross between a wild-type line (Williams 82, AACC) and a low linlolenic acid line (2721, aacc). Individual F_2 seeds were chipped for fatty acid analysis, and the remainder of the seed was germinated for genotype analysis. The average phenotypes of the parent lines (n = 10) are represented in black or white bars, while the averages of all F_2 progeny with the indicated genotypes are represented with gray bars. Error bars represent one standard deviation of the mean. The numbers below the genotypes represent the number of individual seeds that contributed to each phenotype average.

The identified polymorphism allows unambiguous identification of the CX1512-44 derived *GmFAD3C* allele.

Association of GmFAD3 Mutations with the Low Linolenic Acid Phenotype

To investigate the association of the CX1512-44 derived *GmFAD3A* and *GmFAD3C* alleles with seed linolenic acid levels, 107 F_2 progeny of a Williams 82 × 2721 cross were profiled for phenotype and genotype. The line 2721 was an F_6–derived line produced from a cross between Pana and CX1512-44 that had undergone phenotypic selection and was homozygous for the *GmFAD3A* and *GmFAD3C* alleles from CX1512-44 (D. Sleper, personal communication, data not shown). This CX1512-44-derived genotype is represented as aacc, while the corresponding wild-type alleles are represented as AACC. Simple PCR-based assays were developed to distinguish mutant and wild-type alleles at the two loci. F_1 plants were checked for heterozygosity by the identified polymorphisms and grown in growth chambers under defined conditions to produce F_2 seeds. Individual F_2 seeds were chipped for fatty acid analysis and subsequently germinated to allow genotyping from leaf tissue. An unequal, additive association was found for each copy of the mutation and the linolenic acid level (Fig. 3). A stepwise decrease in linolenic acid levels occurred as mutant alleles replaced their wild-type counterparts, with mutations in the *GmFAD3A* gene associating with greater reductions in linolenic acid levels than mutations in the *GmFAD3C* gene, consistent with *GmFAD3A* being the *Fan* locus (Bilyeu et al., 2003). The phenotype of the 2721 parent was completely recovered in the progeny with the aacc genotype. The phenotype of an individual was not sufficiently precise to predict the genotype, even when the standard deviation for phenotype of the genotype group was small.

The overall reduction in linolenic acid levels contributed by mutations in both *GmFAD3A* and *GmFAD3C* was 61 g kg^{-1}, from 89 to 28 g kg^{-1} linolenic acid. The mutation of the two genes thus results in the elimination of over two thirds of the linolenic acid present in the seed. Approximately half of the reduction was obtained from substitutions of a wild-type allele to a heterozygous condition and half from a heterozygous condition to homozygous mutant. By analyzing the differences in average linolenic acid levels for each of the genotypes, the average reduction in linolenic acid levels for both the *GmFAD3A* and *GmFAD3C* mutations was individually determined. As shown in Table 1, the overall average difference in linolenic acid levels between lines with wild-type (AA) or mutant (aa) *GmFAD3A* genes was 46 g kg^{-1} oil while the average differences between wild-type and heterozygous (Aa) or heterozygous and mutant *GmFAD3A* was approximately half the difference from wild type to mutant. The overall average difference in linolenic acid levels was smaller for the *GmFAD3C* gene,

Table 1. Mean difference in seed linolenic acid level for F_2 progeny with allelic substitutions of wild-type (uppercase) and mutant (lowercase) alleles of *GmFAD3A* and *GmFAD3C* at primary locus when alleic combinations at the secondary locus are homozygous or heterozygous. Differences were calculated by subtraction of the linolenic acid level obtained from the first allelic combination at the primary locus minus the linolenic acid level obtained from the second allelic combination (example: AACC to aaCC).

	Primary locus		
Secondary locus	AA to aa	AA to Aa	Aa to aa
	Linolenic acid g kg^{-1} oil		
CC	46.0	24.4	21.7
Cc	47.4	23.6	23.8
cc	43.9	21.0	22.9
	CC to cc	CC to Cc	Cc to cc
AA	17.5	6.5	10.9
Aa	14.2	5.8	8.4
aa	15.3	7.9	7.4

and the differences between heterozygous and either wild type or mutant showed some bias for a larger linolenic acid reduction from Cc to cc than CC to Cc. The range of values for linolenic acid levels in seeds segregating with wild-type alleles was higher than the range for the low linolenic acid mutant allele-containing seeds, even though all seeds were produced under defined conditions in growth chambers. The higher standard deviations for the lines with the highest linolenic acid levels and the relatively low number of F_2 progeny examined (107) does not allow a rigorous testing of the significance of the bias for one allele-contributions.

Soybean lines developed with a *fan* mutation could be enhanced by a further reduction in linolenic acid concentration. To test the ability of the *GmFAD3C* mutation to further reduce linolenic acid levels in a line containing a *fan* mutation, an independent cross was made between the line A5 (*fan fan*) (Rennie and Tanner, 1991) and 2721. The line A5 was previously shown to have a deletion encompassing the *GmFAD3A* gene (Bilyeu et al., 2003; Byrum et al., 1997). Sixty-two F_2 progeny were analyzed for linolenic acid phenotype and genotype. The F_2 seeds were produced under the previously described growth conditions and chipped for fatty acid analysis before germination and genotyping.

As expected for segregation of different mutant alleles at *GmFAD3A*, but both mutant and wild-type alleles at *GmFAD3C*, the overall range of linolenic acid levels was low (25–52 g kg^{-1} oil). For *GmFAD3A*, our assay was only able to distinguish two genotype classes: a class with the deletion of both copies of *GmFAD3A* and a class that was either heterozygous or homozygous for the CX1512-44-derived *GmFAD3A* allele. We assumed the deletion of *GmFAD3A* and the CX1512-44-derived mutant alleles were equivalent, and we could not find significant differences ($P \leq 0.05$) in linolenic acid levels between lines belonging to the different classes (data not shown). Segregation of the mutant *GmFAD3C* gene did produce significant differences in linolenic acid levels, as shown in Table 2. The average difference in linolenic acid from CC to cc was 11 g kg^{-1} oil with heterozygous differences approximately half of that value.

Table 2. Mean difference in seed linolenic acid level for F_2 progeny with allelic substitutions of wild-type (uppercase) and mutant (lowercase) alleles of *GmFAD3C* when *GmFAD3A* is homozygous or heterozygous for allelic deletion or mutant allele. Differences were calculated by subtraction of the mean linolenic acid level obtained from the *GmFAD3C* minus mean the linolenic acid level obtained from the *GmFAD3A* locus (example: aaCC to aacc).

	GmFAD3C		
	Linolenic acid g kg^{-1} oil		
GmFAD3A	CC to cc	CC to Cc	Cc to cc
_ _†	nr‡	nr	4.2
_a or aa	11.2	5.3	5.9

† _ indicates that a *GmFAD3A* allele was deleted.
‡ nr, no lines with _ _ CC genotype were recovered.

To determine the variation in linolenic acid levels in whole seeds derived from lines with specific genotypes for the *FAD3* genes, four selected homozygous F_2 genotypes (aacc, aaCC, AAcc, and AACC) of the Williams 82 × 2721 cross were analyzed for fatty acid phenotype of the F_3 seeds. For each genotype (except aaCC, see Materials and Methods), 10 F_3 seeds produced under our defined growth conditions were individually crushed and analyzed for fatty acid profiles (Fig. 4). The association between the *GmFAD3A* and *GmFAD3C* genotype and the linolenic acid phenotype for the F_3 seeds was consistent with the F_2 analysis. The trend toward higher standard deviations as the linolenic acid levels increased was also apparent in the F_3 analysis. The average linolenic acid level for the 30 aacc F_3 seeds comprised of three sets of 10 seeds produced on separate F_2 plants was 27.3 g kg^{-1} oil, and the standard deviation was 1.8 g kg^{-1} oil.

Another generation of seeds (F_4) representing three independent aacc homozygous genotypes was produced in greenhouse conditions, and whole seeds were analyzed for fatty acids. We had less control of growth conditions in the greenhouse relative to the defined conditions obtained from growth chambers. The low linolenic acid phenotype was sustained in F_4 seeds, with linolenic acid values ranging from 25 to 33 g kg^{-1} oil from individual F_4 seeds with an overall average linolenic acid of 29 g kg^{-1} oil. In seeds derived from the cross between 2721 and Williams 82 that were homozygous for the *GmFAD3A* and *GmFAD3C* mutations, the low linolenic acid phenotype could be identified at the F_2 generation and stably inherited.

DISCUSSION

Here we report the molecular genetic basis for the low linolenic seed trait in soybean line CX1512-44. Using a candidate gene approach, we identified mutations in two of the three soybean microsomal omega-3 fatty acid desaturase genes that associate with a greater than two thirds reduction in seed linolenic acid levels. A compensatory increase in linoleic acid was also associated with mutations in the *FAD3* genes. While the splice-site mutation in *GmFAD3A* almost certainly abolishes the production of a functional enzyme, the biochemical repercussions of the mutation in *GmFAD3C* are less clear. Dissection

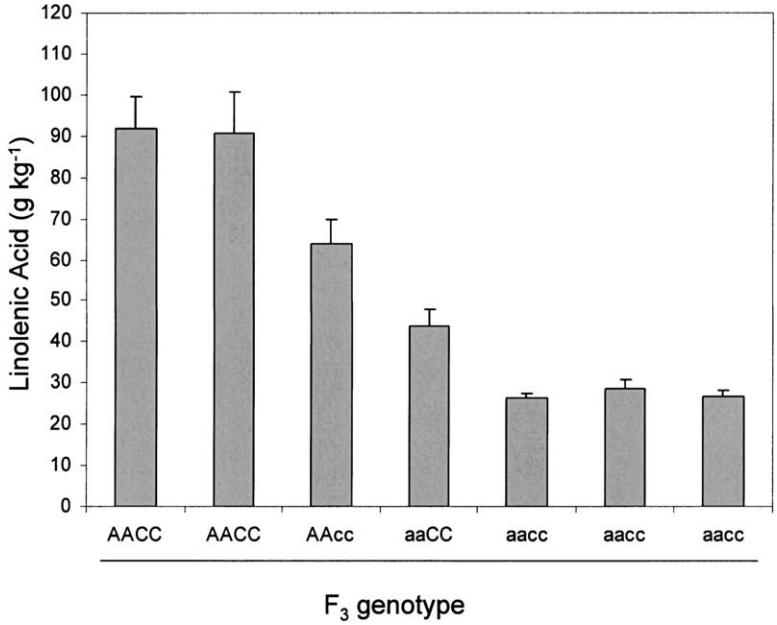


Fig. 4. Phenotype of selected F_3 genotypes homozygous for different combinations of the *GmFAD3A* and *GmFAD3C* wild-type and mutant alleles. Seven lines were selected from genotyped F_2 plants, representing all homozygous combinations of *GmFAD3A* and *GmFAD3C*. For all lines except the aaCC representative (n = 5), 10 independent F_3 seeds were analyzed for linolenic acid. The bars represent the average of the F_3 seeds derived from a single F_2 plant. Error bars represent one standard deviation of the mean.

of the contribution of the two genes *GmFAD3A* (*Fan*) and *GmFAD3C* revealed that the *GmFAD3A* mutation had a greater effect on linolenic acid level reduction than the *GmFAD3C* mutation (linolenic acid levels of 46 g kg^{-1} oil vs. 15 g kg^{-1} oil, respectively), consistent with repeated discovery of mutations at the *fan* locus (Wilcox and Cavins, 1985; Rennie and Tanner, 1991; Rahman et al., 1996). Smaller differences in linolenic acid levels would be much more difficult to detect in a phenotype-based screening program. Our results indicate a gradient of decreasing standard deviations for samples as the linolenic acid level decreases, which may increase the efficiency of phenotypic screening for additional mutations in lines with *fan* mutations (Takagi et al., 1999; Ross et al., 2000).

In most cases examined, the contribution to the decrease in linolenic acid level of the mutant allele in a heterozygote was midpoint between the wild-type and fully mutant genotype. This result would fit well with a model of independent additive enzyme function, where each *GmFAD3* allele represents a proportion of the total potential microsomal omega-3 fatty acid enzyme activity. For Williams 82, each wild-type *GmFAD3A* allele would contribute 23 g linolenic acid kg^{-1} oil to the seed. Assuming the CX1512-44 *GmFAD3C* mutation results in elimination of the encoded enzymes' function, the Williams 82 wild-type *GmFAD3C* alleles contribute 6 g (*Cc* to *CC*) and 9 g (*cc* to *Cc*) linolenic acid kg^{-1} oil to the seed. Similarly, each A5 wild-type *GmFAD3C* allele contributes 5.5 g linolenic acid kg^{-1} to the seed. As part of a breeding strategy, incorporation of the *GmFAD3C* allele from CX1512-44 to soybean lines that have been previously developed with a *fan* mutation would be expected to decrease the linolenic acid concentration an additional 1 to 1.5% (10–15 g kg^{-1} oil), in most cases to about 3% (30 g kg^{-1} oil) linolenic acid, a reasonable target level. The remaining enzyme activity from *GmFAD3B* and the chloroplast-targeted omega-3 fatty acid desaturases could be responsible for producing the residual 3% seed linolenic acid level in segregating populations stabilized for the *aacc* genotype. Reductions of linolenic acid levels to 1% (10 g kg^{-1} oil) have been reported (Ross et al., 2000).

Understanding the molecular basis for the low linolenic acid trait enhances breeding in soybean. Because CX1512-44 contains a novel *fan* mutation in *GmFAD3A* and the first characterized mutation in *GmFAD3C*, breeders can utilize CX1512-44 or its derived lines to further decrease linolenic acid levels in lines containing a *fan* mutation only. In addition, the development of SNP markers specific to the two mutations should allow rapid incorporation of the mutant genes into elite soybean lines, particularly if a backcrossing strategy is adopted.

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REFERENCES

Bernard, R.L., and C.R. Cremeens. 1988. Registration of Williams 82 soybean. Crop Sci. 28:1027–1028.

Bilyeu, K.D., L. Palavalli, D.A. Sleper, and P.R. Beuselinck. 2003. Three microsomal omega-3 fatty-acid desaturase genes contribute to soybean linolenic acid levels. Crop Sci. 43:1833–1838.

Brown, J.W.S., P. Smith, and C.G. Simpson. 1996. Arabidopsis consensus intron sequences. Plant Mol. Biol. 32:531–535.

Byrum, J.R., A.J. Kinney, K.L. Stecca, D.J. Grace, and B.W. Diers. 1997. Alteration of the omega-3 fatty acid desaturase gene is associated with reduced linolenic acid in the A5 soybean genotype. Theor. App. Genet. 94(3–4):356–359.

Fehr, W.R., G.A. Welke, E.G. Hammond, D.N. Duvick, and S.R. Cianzio. 1992. Inheritance of reduced linolenic acid content in soybean genotypes A16 and A17. Crop Sci. 32:903–906.

Konieczny, A., and F.M. Ausubel. 1993. A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific pcr-based markers. Plant J. 4:403–410.

Nickell, C.D., G.R. Noel, T.R. Cary, and D.J. Thomas. 1998. Registration of Pana soybean. Crop Sci. 38:1399.

Rahman, S.M., Y. Takagi, and T. Kumamaru. 1996. Low linolenate sources at the *Fan* locus in soybean lines M-5 and IL-8. Breed. Sci. 46:155–158.

Rahman, S.M., Y. Takagi, and T. Kinoshita. 1997. Genetic control of high stearic acid content in seed oil of two soybean mutants. Theor. Appl. Genet. 95:772–776.

Rahman, S., T. Kinoshita, T. Anai, S. Arima, and Y. Takagi. 1998. Genetic relationships of soybean mutants for different linolenic acid contents. Crop Sci. 38:702–706.

Rennie, B.D., and J.W. Tanner. 1991. New allele at the *Fan* locus in the soybean line A5. Crop Sci. 31:297–301.

Ross, A.J., W.R. Fehr, G.A. Welke, and S.R. Cianzio. 2000. Agronomic and seed traits of 1%-linolenate soybean genotypes. Crop Sci. 40:383–386.

Shanklin, J., E. Whittle, and B.G. Fox. 1994. Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. Biochemistry 33:12787–12794.

Simpson, C.G., G.P. Clark, J.M. Lyon, J. Watters, C. McQuade, and J.W.S. Brown. 1999. Interactions between introns via exon definition in plant pre-mRNA splicing. Plant J. 18:293–302.

Takagi, Y., S.M. Rahman, T. Anai, S.K. Wasala, T. Kinoshita, and M. Khalekuzzaman. 1999. Development of a reduced linolenate soy mutant by re-irradiation and its genetic analysis. Breed. Sci. 49:1–5.

Wilcox, J.R., and J.F. Cavins. 1985. Inheritance of low linolenic acid content of the seed oil of a mutant in *Glycine max*. Theor. Appl. Genet. 71:74–78.

Yadav, N.S., A. Wierzbicki, M. Aegerter, C.S. Caster, L. Perez-Grau, A.J. Kinney, W.D. Hitz, Jr., B. Booth, Jr., K.L. Schweiger, S.M. Stecca, M. Allen, R.S. Blackwell, T.J. Reiter, S.H. Carlson, K.A. Russell, J. Feldmann, J. Pierce, and J. Browse. 1993. Cloning of higher plant omega-3 fatty acid desaturases. Plant Physiol. 103: 467–476.

Ye, J., E.J. Parra, D.M. Sosnoski, K. Hiester, P.A. Underhill, and M.D. Shriver. 2002. Melting curve SNP (McSNP) genotyping: A useful approach for diallelic genotyping in forensic science. J. Forensic Sci. 47:593–600.